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Hepatitis C Plasma Viral Load Is Associated With HCV Genotype But Not With HIV Coinfection

A. Berger, M. v. Depka Prondzinski, H.W. Doerr, H. Rabenau, and B. Weber

Institut für Medizinische Virologie, Zentrum der Hygiene (A.B., H.W.D., H.R., B.W.), and Angiologische Ambulanz, Zentrum der Inneren Medizin (M.v.D.P.), Universitätskliniken Frankfurt, a. M., Germany

The influence of human immunodeficiency virus (HIV) coinfection and hepatitis C virus (HCV) genotype distribution on HCV viral load and alanine amino transferase (ALT) levels in chronically infected patients remains unclear. In the present study, serum samples from a group of haemophiliac patients were investigated retrospectively. HCV geno- and subtyping was carried out using the Inno line probe assay (Inno LIPA, Innogenetics, Zwijnaarde, Belgium) in 87 patients positive by HCV RT PCR. Of these patients, 31 (35.6%) were HIV coinfected. HCV RNA was quantified with the HCV Monitor kit (Roche, Basel, Switzerland) in 43 patients (22 HIV-negatives, 21 HIV-positives). The most prevalent genotypes were 1 (n = 52) and 3a (n = 16) followed by genotype 2 (n = 9) and 4 (n = 3). Mixed infections were detected in 7 patients. Of genotype 1 positive samples, 24 and 23 were classified as subtype a and b, respectively. Five samples could not be subtyped. Although higher mean values of ALT were observed in genotype 1 infected patients, there was no statistically significant association between HCV genotype or subtype and liver enzymes ($P > 0.05$). On the other hand, statistically significant higher HCV RNA titres were observed in haemophiliacs infected with HCV genotype 1 in comparison to those infected with other genotypes ($P < 0.01$). No relationship was found between the presence of HIV coinfection and viral load of HCV RNA. There was no evidence that HCV infection had a more severe outcome in HIV-positive patients who had been infected with HIV and HCV more than ten years ago, even in those with very low CD4+ cell counts. No clear association between high ALT levels and large amounts of viral RNA was observed. In conclusion, a large viral load is associated with HCV genotype 1 infection; HIV coinfection has no clear effect on the intensity of HCV replication. An ongoing prospective study will evaluate the respective role of viral load, genotype, HIV coinfection and ALT level in the response to interferon therapy.

KEY WORDS: HCV RNA, HCV genotypes, quantitation, PCR

INTRODUCTION

Hepatitis C virus (HCV) is the major causative agent of transfusion-associated and sporadic non-A non-B hepatitis worldwide. Prior to the introduction of blood donor screening for anti-HCV and inactivation methods for pooled plasma products, nearly all haemophiliac patients became infected with HCV, because virtually all clotting factor concentrates were contaminated [Makris et al., 1993]. The majority of haemophiliacs transfused with clotting factor concentrates between 1978 and 1984 also became infected with human immunodeficiency virus (HIV) [Eyster et al., 1993]. The population of haemophiliac patients treated at the University Clinics of Frankfurt shows a HCV and HIV seroprevalence of 77.4% and 23.4%, respectively [Weber et al., 1995].

The HCV genome shows considerable degrees of variation. At least six HCV genotypes with two or more related subtypes, according to the classification system based on the nonstructural gene 5 (NS5) sequence analysis of Simmonds et al. [1993], have been identified. Types 1, 2 and 3 are distributed almost worldwide [McOmish et al., 1994]. In western European countries, 50% of anti-HCV-positive blood donors are infected with HCV type 1, 40% with type 3 and 10% with type 2; a similar distribution is observed in Australia. In Japan, 70–80% of the HCV positives are infected with type 1 and the remainder with type 2. HCV type 3 has not been found in Japan.

Distinction of HCV genotypes is important since the outcome of chronic infection and response to antiviral therapy with interferon correlates with the genotype. Genotype 1, especially subtype 1b, has been associated with more severe chronic liver disease and poor response to interferon therapy [McOmish et al., 1994; Yoshioka et al., 1992]. The possibility that the viral titre may influence the likelihood of achieving remission after interferon therapy has been suggested [Cees et al., 1994]. Kobayashi et al. [1993] found that pretreatment viral titre was more important in predicting response to interferon than was genotype. Other variables, i.e., age and duration of disease, reinfection or coinfection with differ-

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Address reprint requests to Dr. Bernard Weber, Laboratoire Lieners et Hastert, Rue de l'Hôpital 18 L-9244 Diekirch, Luxemburg.

ent HCV genotypes or subtypes, may be independent predictors of response [Dusheiko et al., 1994]. Individuals can be coinfected with different genotypes particularly if they have experienced multiple exposures to HCV such as haemophiliac patients. In HCV-HIV coinfected individuals, liver disease may be more aggressive than in HIV-negative patients [Jarvis et al., 1994; Eyster et al., 1993].

In the present study, the HCV genotype distribution and viral load in a population of haemophiliacs with or without HIV coinfection were investigated. The aim was to evaluate the influence of HIV coinfection and HCV genotype distribution on HCV viral load and ALT levels. Furthermore, CD4+ cell count was determined in HIV-coinfected patients in order to estimate the relationship between HCV viral load and immunosuppression.

MATERIALS AND METHODS

The study population consisted of 87 haemophiliacs (positive for HCV antibodies by a second generation test, and HCV RNA) who were followed-up in regular intervals at the University Clinics of Frankfurt/M. The mean age of these patients was 40.5 years (range 24–73 years). HCV genotype was determined with a commercially available detection system (Inno-LIPA, Innogenetics). Thirty-one patients (35.6%) were coinfected with HIV. All these patients had been infected with HCV and HIV before 1984. The average CD4+ cell count of HIV-positive patients was 325 cells/μl (range 9–930). Serum samples were stored after collection at −70°C prior to testing. In a subgroup of 43 patients (22 HIV-negatives, 21 HIV-positives) HCV RNA was quantified with the HCV monitor assay.

HCV Genotyping

HCV genotyping was carried out using a type-specific detection system of the PCR-amplified 5′-noncoding region (Inno-LIPA, Innogenetics, Zwijnaarde, Belgium). The Inno-LIPA was carried out according to the manufacturer's instructions. Briefly, viral RNA was extracted from serum and reverse-transcribed into cDNA, followed by an amplification step with primers complementary to the conserved areas of the 5′-noncoding region of the different HCV types. During amplification, biotinylated primers were incorporated in the amplified DNA fragments. Specific oligonucleotide probes immobilized as parallel lines on membrane strips were hybridized with amplified sample material. After hybridization, streptavidin labelled with alcaline phosphatase was added and became bound to any biotinylated hybrid previously formed. Incubation with nitroblue tetrazolium/4-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) chromogen resulted in a purple/brown precipitate. The reactivity of an amplified fragment with one or more lines on the strip allowed the easy recognition of HCV genotypes.

Quantitative Competitive Polymerase Chain Reaction (PCR)

The viral load of 43 samples was determined using the HCV Monitor test (Hoffmann-La Roche, Basel, Switzerland). This assay includes a quantitation standard (QS) that is coamplified with the target, to monitor the efficiency of the amplification reaction. Briefly, HCV target RNA and QS RNA were reverse-transcribed and amplified (primers selected from the highly conserved 5′-noncoding region of the HCV genome) in a single tube reaction using rTth polymerase. After amplification, the products were denatured, transferred to a microwell detection plate, and diluted serially twice and hybridised to HCV-specific microwells or to QS-specific microwells. The highest dilution that gave an extinction (A_{450}) between 0.5 and 2.0 on the HCV-specific well and on the QS-specific wells were then selected and used to calculate the number of RNA copies/ml in the sample according to the following formula:

$$\frac{\text{OD sample} \times \text{DF sample amplicon}}{\text{OD QS} \times \text{DF QS amplicon}} \times \text{DF extraction} \times \text{QS copies} = \text{HCV RNA copies/ml}$$

(DF: dilution factor, OD: optical density).

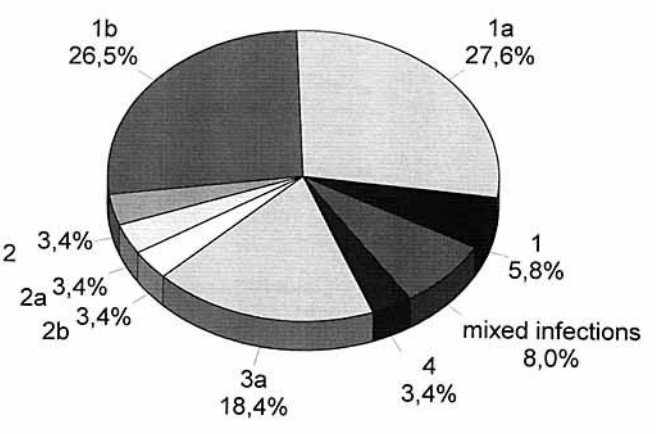

Fig. 1. HCV genotype distribution in haemophilic patients (n = 87) treated at the University Clinics of Frankfurt/Main.

Statistics

Wilcoxon U test was used to determine the statistical significance of differences between groups. Spearman rank order correlations were calculated for HCV RNA level, liver enzyme levels and CD4+ lymphocyte count.

RESULTS

The HCV geno- and subtype distribution is shown in Figure 1. The present study revealed that HCV genotype 1 was the predominant genotype (59.1%), followed by genotype 3 (18.2%) and 2 (10.2%). In 3 (3.4%) cases, genotype 4 was found. Mixed infections were detected in 7 patients (7.9%); 5 of these patients were HIV-positive. HCV subtypes 1a and 1b were detected in 24 (27.3%) and in 23 cases (26.1%), respectively. In five samples (5.7%) subtyping was not possible. Genotype 2a was found in 3 and 2b also in 3 (3.4%) patients. In 3 samples subtyping of genotype 2 could not be achieved. All genotype 3 infections were of subtype 3a.

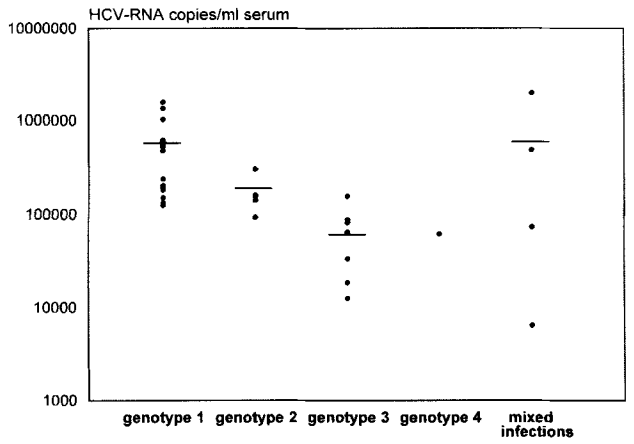

Fig. 2. HCV-RNA copies/ml serum in haemophilic patients infected with different HCV genotypes.

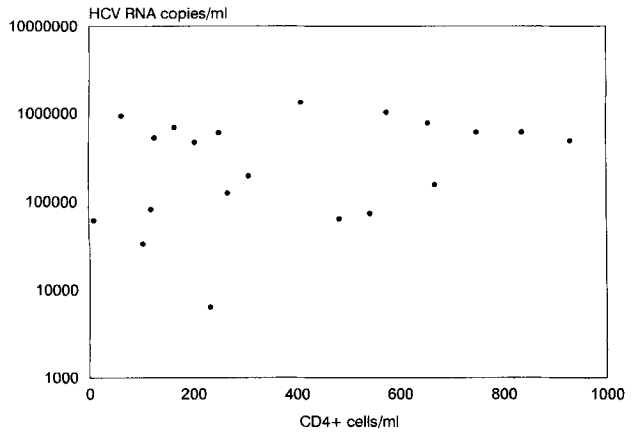

Fig. 3. HCV-RNA copies/ml serum in relation to CD4+ cell count in HCV coinfected HIV-positive haemophiliacs (n = 20).

Thirty-one of the 87 patients were coinfected with HIV. No differences were noted in the distribution of HCV genotypes in HIV-infected individuals in comparison to the HIV-seronegatives (data not shown). The average viral load of the 43 patients determined with the HCV monitor test was 4.06×10^5 copies RNA/ml (range: 6.7×10^2 to 2.0×10^6). The viral titre was significantly higher in patients with the HCV genotype 1 than in genotype 3 infections ($P = 0.000009$) and in genotype 2 infections compared with genotype 3 infections ($P = 0.0109$). Figure 2 shows that there was, although statistically not significant, a trend towards lower viral titres in genotype 2 than in genotype 1 infections. No significant difference was observed in the average viral load nor in the mean value of liver enzymes at the time of sample collection between HCV- and HCV/HIV-coinfected patients ($P = 0.26$). In HIV-coinfected patients no significant correlation was found between HCV RNA levels and CD4+ counts ($P = 0.5$) (Fig. 3).

The mean serum aminotransferase concentrations

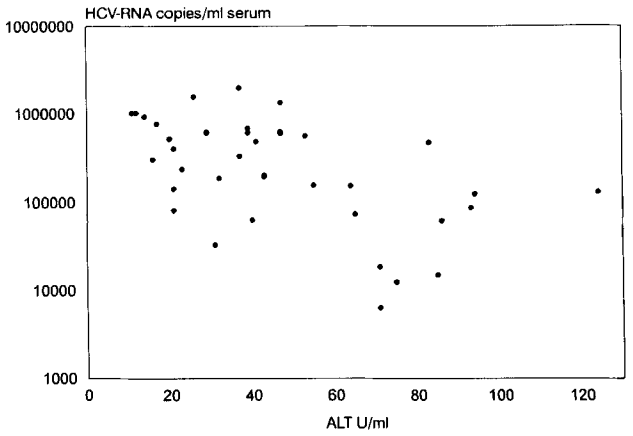

Fig. 4. Liver enzyme levels according to genotype distribution in haemophilic patients (n = 46).

were not significantly different ($P = 0.2$) between the various HCV genotypes. There was a trend towards lower liver enzyme levels in genotype 2 infected patients (Fig. 4).

DISCUSSION

The overall genotype distribution in haemophiliac patients of the University Clinics in Frankfurt a. M. is very similar to that reported in other high risk groups and blood donors [Hofmann et al., 1995; McOmish et al., 1994; Roggendorf et al., 1993]. It has been suggested that some genotypes might be associated with severity of the disease and higher viral titres [Dusheiko et al., 1994]. Our analysis shows significantly higher viral titres in patients infected with HCV genotype 1 in agreement with other studies [Yoshioka et al., 1992]. As shown by Pozzato et al. [1994], a poor prognosis of HCV infection is associated with genotype 1b. McOmish et al. [1993] reported higher serum ALT concentrations in HCV infections caused by some genotypes than in other types. This finding was not confirmed in the present study and it may reflect the fact that serum aminotransferase levels at one point in time are poor indicators of the long-term natural history of HCV infection. In a previous study by Dubois et al. [1994], there was no association between HCV RNA levels and hepatic enzyme levels. The existence of relatively conserved patterns of substitutions in the 5′-noncoding region that are characteristic of different HCV types provides useful "signature" sequences for the identification of HCV genotypes using genotype- or subtype-specific probes. Subtyping of HCV isolates can be carried out by sequence analysis or hybridization with type- or subtype-specific probes (i.e., Inno-LIPA). A relatively good agreement was observed between Inno Lipa and sequencing of the 5′-noncoding region [Andonov and Chaudhary, 1995; van Doorn et al., 1994]. In this study, subtyping could not be obtained in 8 samples. A possible explanation could be the presence of a mutation in the genome region complementary to the subtyping probe. A further hy-

pothesis for the lack of discrimination between subtypes 1a and 1b could be the presence of very low amounts of viral RNA under the detection limit of the line probe assay in these samples. However, the failure of subtyping of 3 of these samples could not be explained by a lack of sensitivity of the detection system because subtyping was possible in other samples with an equivalent or even lower viral load.

Eyster et al. [1993] suggested that HIV or its therapy may accelerate liver failure in HIV-positive adults with haemophilia. It was also suspected that HIV infection may potentiate the liver injury in chronic non-A non-B hepatitis or hepatitis C [Eyster et al., 1994; Martin et al., 1989; Telfer et al., 1994a]. Filippo et al. [1991] observed severe chronic HCV hepatitis in 8 of 65 HIV-positive haemophiliacs, compared to none of 105 HIV-negative haemophiliacs. Among coinfected haemophiliacs with liver failure, the majority have low CD4 counts, lymphocytopenia and thrombocytopenia. In contrast to these reports, in our population of haemophiliacs no differences were observed in terms of mean liver enzyme activity values and viral load between HIV-negative and HIV-coinfected HCV-infected haemophiliacs. Moreover, there was no association between the stage of HIV infection, as shown by CD4+ cell counts, and HCV plasma viremia. Although Telfer et al. [1994] speculated that HCV replication is enhanced in the presence of immune deficiency, they found no association between progression of HCV infection and CD4 count. Sherman et al. [1993] observed significantly higher HCV RNA titres in HIV-coinfected patients. However, in our patients immune status as defined by CD4+ count was not associated with different viral titres. There was no evidence that HCV infection had a more severe outcome in HIV-positive patients who had been infected more than ten years ago, even in those with very low CD4+ cell counts (Fig. 3).

The issue of genotype pathogenicity in a disease such as chronic HCV infection is complex. Other host and viral factors will also influence the outcome. Disease severity may be related to the duration of infection. Viral load at the time of inoculation, most of acquisition, reinfections, host immunity, genetic factors, age, coexistent viral and parasitic infections and alcohol abuse may determine the outcome of infection [Dusheiko et al., 1993].

The recent identification of important variations in even the most "conserved" 5′-noncoding region of the HCV genome has posed a major challenge to quantitation of HCV RNA with reverse transcriptase (RT-PCR) and branched DNA (bDNA) detection. The bDNA assay of the first generation was shown to underestimate RNA levels in patients with HCV genotype 3 infection [Collins et al., 1995; Lau et al., 1995]. Reliable data are not available on the influence of genomic variation and amplification rate with RT-PCR. If quantitation of HCV RNA by RT-PCR is associated with HCV genotype, the true relation between HCV viraemia and the clinical and virological outcome should be reconsidered.

The clinical evaluation of genotype virulence remains difficult. Many patients will need to be studied carefully with appropriate definitions of virological response and prolonged follow-up. In an ongoing prospective study, the influence of viral load and genotype on the response to interferon therapy will be evaluated in a selected group of 30 haemophiliac patients.

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